Table 12. Incidence of Skeletal Variations and Anomalies in F_1 Fetuses from a Segment II Reproductive Toxicity Testing in ~~Rats~~ Rabbits

Treatment	Vehicle	r-Hirudin		
Dose (mg/kg)	0	1	10	30
Number of Litters Evaluated	15	15	14	15
Number of Fetuses Evaluated	133	128	117	93
	No. of Fetuses/No. of Litters Affected			
Skeletal Variations				
-Sternebra (Reduced Ossification or Unossified 5th and 6th)	52/14	41/14	34/11	34/13
-Ribs (presence of 13th rib)	94/15	60/13	70/13	58/14
Skeletal Anomalies:				
-Skull (Hyoid) Reduced Ossification	15/7	3/2	14/3	7/3
-Sternebra (Reduced Ossification)	0/0	3/3	1/1	2/2
-Hind limbs (Talus) Reduced Ossification or Unossified	4/3	2/2	2/1	4/3
-Pelvis (Pubic Bone) Reduced Ossification or Unossified	8/4	9/4	7/4	8/4
Skeletal Malformations:				
Sternebra (fused)	0/0	2/2	0/0	1/1
Ribs (Wavy)	0/0	1/1	0/0	0/0

Note: Variations/anomalies both refer to non-permanent structural changes which have no obvious detrimental effects. Changes classified as variations occurred at a > 10% incidence in the historical population. Finally, variations/anomalies which occurred only at the mid or low doses or which occurred at a greater incidence in the control groups are not depicted above.

As is shown in Tables 11 and 12, treatment with r-Hirudin neither increased the incidence of soft tissue anomalies/malformations nor the incidence of skeletal variations, anomalies or malformations, relative to vehicle-treated control groups.

In conclusion, r-Hirudin was not teratogenic nor maternally toxic at i.v. doses of 1, 10, and 30 mg/kg tested herein. However, at the 30 mg/kg dose r-Hirudin was with increased number of early resorptions, increased post implantation loss and resulting decreased litter size observed. The no effect dose in terms of embryo toxicity was 10 mg/kg.

I.V. Segment II/III. Perinatal and Postnatal Study in Rats (Study # 12506RSR)

Study Started: March 6, 1995

Study Completed: December 13, 1995

GLP Requirements: A Statement of Compliance with GLP regulations was included.

Test Species: Pregnant Sprague Dawley Crl:CD(SD)BR rats.

No. of Animals: 25/group.

Route of Administration: I.V.

Drug Batch No.: 115011

Methods: Pregnant Sprague Dawley rats (25/group) were given i.v. doses of 0 (vehicle: 0.9% saline), 1, 10 and 30 mg/kg/day or r-hirudin from day 6 of gestation to day 21 of lactation (covering the period of Segment II and Segment III). Additionally, 6 females/group were included in this study and treated in similar fashion for monitoring plasma drug levels (data will be reported separately by the sponsor). All dams were observed daily for clinical signs and mortality. Body weights and food intakes were recorded at designated intervals. All dams were allowed to deliver spontaneously and raise their offspring. The number of live/dead pups were recorded, and live pups were weighed and sexed. During lactation period, growth and differentiation of the pups were observed and development parameters were assessed. On day 21 of lactation, one or two male and female pups per litter were selected for F_1 generation study. At sexual maturity, F_1 male and female rats from the same group were continuously mated and were killed on day 20 of gestation and their contents were examined.

Results: Seven dams from high dose group died/killed during gestation period, generally just before or at the time of delivery; additionally, 4 females from the same group died during lactation period. Even though no factors contributing to death were established, all deaths were considered by sponsor to be treatment related. Additionally, there were 4 deaths (2 in control group and 2 in low dose group) which were considered to be not treatment related. Treatment had no significant effect on body weight gains and food intakes. The duration of gestation was comparable in all groups. Number of pups delivered, pups

weights, sex ratio and physical development were comparable in all groups, except increased incidence of dilation of renal pelvis was seen in pups of high dose group which were sacrificed at weaning (pup incidence: control = 0%, low dose = 3%, mid dose = 0.4% and high dose = 12%). No treatment related effects were seen in F_1 generation. The fertility of F_1 males and females, and prenatal development of F_2 generation fetuses were not affected by the treatment of p-generation dams.

Effects on Segment II/III. Perinatal and Postnatal Study in Rats

Parameters	Control	Low Dose	Mid Dose	High Dose
Total Mated	25	25	25	25
# of Pregnant	21	20	23	25
% Pregnant	84.0	80.0	92.0	100.0
# of Pregnant Surviving Delivery	21	19	23	15
Length of Gestation (days)	21.2	21.4	21.1	21.2
# of Live Pups/Dam on Day 1	11.5 ± 3.7	13.3 ± 3.5	13.7 ± 2.2	12.2 ± 3.4
Pups Surviving at Day 4 (%)	98.3	97.6	96.8	94.0
Pups Surviving at Day 21	95.4	99.2	99.7	95.9
Mean Pups Wt. (g)				
Day 1	6.8 ± 0.7	6.4 ± 0.5	6.4 ± .6	6.6 ± 0.8
Day 21	39.7 ± 8.1	36.7 ± 4.9	37.7 ± 5.4	41.0 ± 7.0
Sex Ratio (% males)				
Day 0	56.2	49.2	46.8	51.6
Day 21	56.4	49.6	46.2	49.7

The highest tested dose produced maternal toxicity (11/25 dams died/killed in delivery and early post-partum period). However, no significant adverse effect on reproductive parameters were seen in rat following i.v. administration of up to 30 mg/kg/day of r-hirudin during perinatal and postnatal period. No treatment related macroscopic abnormalities were seen in females which died, nor in scheduled sacrificed animals.

GENETIC TOXICOLOGY:

R-Hirudin: Microbial Mutagenicity Study (Ames Test) (Report # 88.1571)

Study Started: September 21, 1988

Study Completed: October 7, 1988

GLP Requirements: A Statement of compliance with the GLP regulations and quality assurance unit was included.

Drug Batch No.: U001

Methods: The mutagenic activity of R-Hirudin, at concentrations of 4, 20, 100, 500, 2500, and 5000 µg/plate, was tested using the AMES test (tester strains Salmonella typhimurium TA100, TA1535, TA1537, TA1538, TA98 and E.Coli WP2uvrA with and without an S-9 fraction liver homogenate metabolizing system. Test concentration selection was based on dose range finding experiment with all the said strains an in which no toxicity was observed at the 5000 µg/plate high dose. Thus, the 5000 µg/plate concentration was selected as the high dose for the study. Positive controls; without metabolic activation: Na-azide (TA100 and TA1535), 9-Amino acridine (TA1537); 2-Nitrofluorene (TA98 and TA1538); N-methyl-N-nitro-N-Nitrosoguanidine (MNNG; WP2uvrA) and with metabolic activation: Benzo[a]pyrene (TA100, TA1535, TA1537, TA1538, TA98, and WP2uvrA) and 2-aminoanthracine (TA100, TA1535, TA1537, TA1538, TA98, and WP2uvrA) were also tested in order to insure test viability. Criteria for a positive mutagenic effect were not indicated.

Results: Results from the toxicity tests showed that r-Hirudin at concentrations ranging from 4 to 5000 µg/plate was not toxic to any of the bacterial strains tested. R-Hirudin did not produce a significant increase in the number of revertant colonies either in the presence or absence of the S-9 Mix and no dose-dependent effect was observed. Background control and positive control compounds produced the expected number of colonies, insuring the validity of the experiment.

In conclusion, r-Hirudin at concentrations up to 5000 µg/plate tested negative for mutagenicity in the Ames assay.

The above Ames test was repeated (study # 95.0264) using final drug product and the result was negative.

Unscheduled DNA Synthesis Test (UDS) with r-Hirudin in an A 549 Mammalian Cell Line (Report No, 88.2085)

Study Started: October 27, 1988

Study Completed: December 6, 1988

GLP Requirements: A Statement of compliance with the GLP regulations and quality assurance unit was included.

Drug Batch No.: U001

Methods: The ability of r-Hirudin (1, 3, 10, 30, 100, 300, and 1000 µg/ml) to induce unscheduled DNA synthesis in mammalian cells (A 549 human cell line) was investigated by determination of excision repair, in the presence and absence of a fraction of rat liver homogenate (S-9) for metabolic activation. The 1000 µg/ml high dose was selected as a maximal dose at which no precipitation occurred. Vehicle and positive controls: 1 µg/ml of NQO (4-Nitroquinoline-N-oxide) with metabolic activation and 10 µg/ml of BP (benzo(a)pyrene) without, were also examined in order to validate the experiment. Briefly, cells were pretreated with 10 mM hydroxyurea and reduced fetal calf serum (2%) in order to reduce or inhibit semi-conservative DNA replication. Following 3 hours of incubation with r-Hirudin or control substances, DNA content and the incorporation of ^{3}H-Thymidine into DNA were determined using a colorimetric assay and liquid scintillation counting, respectively. Criteria for a positive test were not indicated.

Results: R-Hirudin at concentrations ranging from 1 to 1000 µg/ml was soluble in the culture media and produced no significant cytotoxic effects in the A 549 mammalian cell line. In the second test all r-Hirudin-treated groups showed a small increases in thymidine incorporation (relative to control values of 1661 dpm/microgram DNA) in the absence of the S-9 fraction. Although these differences were statistically significant at the 3.0 (22%) and 30 µg/ml doses (38%), differences were not dose-dependent and were not evident in two other independent studies conducted in the absence of the S-9 fraction. In all three studies conducted in the presence of the S-9 fraction, r-Hirudin produced no statistically significant increases in thymidine incorporation relative to vehicle controls. The positive controls produced the expected increases in thymidine incorporation, thus insuring the validity of the study.

In conclusion, r-Hirudin at concentrations up to 1000 µg/ml tested negative for induction of DNA synthesis in the A549 mammalian cell line under the conditions of the assay.

Test for induction of Chromosomal Aberrations by r-Hirudin In Vitro in V79 Chinese Hamster Cells. (Report No. 90.0325)

Study Started: February 19, 1990

Study Completed: April 2, 1990

GLP Requirements: A Statement of compliance with the GLP regulations and quality assurance unit was included.

Drug Batch No.: C 004

Methods: Chinese hamster cells V79 were treated with r-Hirudin at concentrations of 200, 500, and 5000 µg/ml for periods of 4 hours in the presence and absence of a S-9 liver homogenate metabolic activation system. The 5000 µg/ml dose was indicated to be the highest concentration tolerated for the test system. Both negative and vehicle controls and positive controls: Ethylmethanesulfonate (EMS; 2000 µg/ml, without metabolic activation) and Cyclophosphamide (CPA; 5 µg/ml, with metabolic activation) were included to insure test validity. Colcimide (0.04 µg/ml) was added at 4.5, 15.5, and 25.5 hours after the start of treatment and the cells were harvested 2.5 hours later. 100 metaphase cells/experimental group were examined for chromosome and chromatid-type aberrations (i.e. gaps, breaks, fragments, minute, deletions, exchanges, dicentrics, chromosome disintegrations, and ring formations). Finally, metaphase cells with 5 or more aberrations were classified as multiple aberrations. A test agent was considered positive for mutagenicity if it produces a significant increase in the rate of aberrations compared to the negative control at one concentration tested or if there is a reproducible concentration-related increase in the aberration rate.

Results: R-Hirudin at a dose of 5000 µg/ml did not reduce the mitotic index by greater than 20% relative to solvent controls and likewise produced no relevant increase in the number of polyploid cells. Finally, r-Hirudin produced no relevant, reproducible increase in the incidence of aberrations above the range of solvent controls (exclusive of gaps) at any of the concentrations tested. Treatment with the positive controls, EMS and CPA, resulted in expected increases in the percent of aberrant cells (30 and 20%, relative to 1.5 and 2% in the respective solvent controls) in the presence and absence of the S-9 fraction, respectively.

In conclusion, r-Hirudin tested negative for mutagenic effects in the chromosome aberration test system in vitro using the V79 Chinese Hamster cell line.

The above in vitro chromosomal aberration test was repeated (study # 95.0266) using final drug product, and cells were harvested at 20, 28 and 48 hr (in the absence of S-9 mix) after the start of treatment (cells in the presence of S-9 mix were treated only for 6 hours then washed and incubated for additional 14 or 22 hours). Irrespective of the presence or absence of metabolic activation, treatment with r-hirudin did not produce any significant reproducible increase n chromosomal aberration over the value obtained for the control group. The positive control gave expected results. Thus, r-hirudin had no clastogenic activity in this in vitro cytogenetic test.

V79/HGPRT Mammalian Cell-Forward Gene Mutation Assay
(Study # 95.0281)

Testing Laboratories: Pharma Development Corporate Toxicology Hoecht,
Frankfurt am Main
Germany

Study Started: February 15, 1995

Study Completed: May 15, 1995 (report date)

Strain Employed: V79 Chinese hamster lung fibroblasts

Solvent Control: Saline.

Positive Control: Ethylmethanesulfonate (EMS: 8 mM) and 9,10-dimethyl-1,2-benzanthracene (DMBA: 30 μM).

Source of Metabolic Activation: Rat liver microsomal enzymes.

Drug Batch No.: 116011

Criteria of Genotoxic Effect: Mutation frequency in the exposed culture must be at least triple the mean mutation frequency of the negative control cultures, and effect must be dose related and reproducible.

Results: Sponsor has conducted 2 separate experiments. Irrespective of the presence or absence of S-9 mix, drug was not mutagenic in this test. Positive control gave the expected results. Thus, r-hirudin had no mutagenic potential in V79/HGPRT forward gene mutation assay.

Mouse Micronucleus Test
(Study # 95.0250)

Testing Laboratories: Pharma Development Corporate Toxicity
Hoecht,
Frankfurt am Main
Germany

Study Started: February 20, 1995

Study Completed: May 31, 1995

Test Species: NMRI mouse (both sexes)

No. of Animals: 5/sex

Route of Administration: I.V.

Dose Levels: 100 mg/kg

Drug Batch No.: 116011

Basis of Dose Selection: Not given.

Solvent Control: 0.9% saline.

Positive Control: Endoxan (20 mg/kg, i.v.).

Methods: Animals were given a single i.v. dose of r-hirudin at 12, 24 or 48 hr prior to sacrifice and preparation of the bone marrow (positive control mice were sacrificed t 24 hr after Endoxan administration). On the Giemsa-stained slides, 1000 polychromatic erythrocytes per animal were examined for the presence of micronuclei.

Results: r-Hirudin did not induce an increase of micronucleated polychromatic erythrocytes in mice bone marrow. Positive control gave expected results. Thus, r-hirudin is not mutagenic in this test system.

Special Toxicity Studies:

Local Toxicity of r-Hirudin Following Intravenous, Intraarterial, and Perivenous Administration in Rabbits (Report Numbers 134-24, 134.2-24, 134.24.1, 134.24.2, 134.2-24.3, 134.2-24.1, 134.2-24.2)

Study Started: Report #134-24: 1/31/89; #134.2-24: 5/18/89; #134.24.1: 1/31/89; #134.24.2: 5/16/89; #134.2-24.3: 5/18/92; #134.2-24.1: 5/18/92; #134.2-24.2: 5/18/92

Study Completed: Report #134-24: 4/17/89; #134.2-24: 7/15/89; #134.24.1: 4/14/89; #134.24.2: 8/30/89; #134.2-24.3: 7/13/92; #134.2-24.1: 7/14/92; #134.2-24.2: 7/15/92

GLP Requirements: Statements of compliance with GLP regulations were included in all studies.

Animals: Rabbits; house strain; approximately 12 weeks of age; weighing between 2.0 and 2.5 kg.

Drug Lot No.: #13, #Ch.-B U 001, #Ch.-B U 003, and #C 005

Methods: Studies in rabbits examined the local tolerance of r-Hirudin was studied in rabbits following i.v., i.a., s.c., and/or perivenous injections. A brief summary of the methods used in the aforementioned studies are presented below:

In the first study (Report No. 134-24) male and female rabbits (4/sex/route of administration) were injected with r-DNA hirudin (Lot No Ch.-B U 001) or vehicle (0.9% NaCl) into opposite ears, as either a single bolus intravenous (25 mg in 0.5 ml), intraarterial (25 mg in 0.5 ml) or perivenous (5 mg in 0.1 ml) dose. Repeat i.v., i.a., and perivenous studies were also conducted in rabbits (Report Nos. 134.2-24, 134.2-24.1, and 134.2-24.2, respectively). In the repeat i.v. study, 10 male rabbits were injected with r-Hirudin (Lot No. 13) or vehicle (aqua ad injectibilia) as a single 25 mg i.v. bolus dose in a total volume of 0.5 ml. In the repeat i.a. and perivenous studies, r-Hirudin (Lot No. 13) was dissolved in either 0.9% Nail (i.a. study) or in aqua ad injectabilia (perivenous study) and injected at a dose of 25 mg in 0.5 ml (i.a.) or perivenously into the ear at a dose of 5 mg in 0.1 ml. Animals in the initial studies were evaluated immediately for clinical effects and were sacrificed at 24 and 48 hours after dosing for histological evaluation of the injection sites (2 rabbits/sex/time point). Rabbits in the repeat studies underwent histological evaluations of the injection sites following sacrifice at 2 and 3 days after the i.v. and i.a. injections and at 3 and 8 days after the perivenous injection.

In three other studies in rabbits the local toxicity of r-Hirudin was investigated following subcutaneous administration of r-Hirudin into the right or lateral abdominal skin. In the first study (Report No. 134.24.1), two rabbits/sex were subcutaneously injected with single doses of r-Hirudin (Lot No. U 001; dissolved in 0.9% Nail) at doses of 6.25, 12.5, 18.75 and 25 mg in a total volume of 0.5 ml at different injection sites. In the second study (Report No. 134.24.2), rabbits (2/sex) were injected with r-Hirudin (Lot Nos. U 003 and C 005; dissolved in aqua ad injectabilia) at a doses of 5, 12.5, 25, 37.5 and 50 mg in a volume of 0.5 ml at different injection sites. Finally, in the third study (Report No. 134.2-24.3), 10 male rabbits were administered a s.c. injection of r-Hirudin (Lot No. 13; dissolved in aqua ad injectabilia) at a dose 25 mg in a total volume of 0.5 ml. Rabbits from the three studies were sacrificed at 2 days (1st and 2nd studies) and at 3 and 8 days (5 rabbits on each day in the 3rd study) and underwent histological examination of the injection sites.

Results: In the initial and repeat studies, i.v. and perivenous injection of r-Hirudin at doses of 25 mg/0.5 ml (i.v.) and 5 mg/ 0.1 ml (perivenous injection) produced clinical signs of slight to moderate perivascular redness and histological findings of focal bleeding in up to half of the animals tested. In the two intraarterial local toxicity studies, r-hirudin (25 mg in 0.5 ml) produced clinical signs of unrest (indicative of irritation, initial study only) and slight to marked perivascular redness histological findings of slight to marked to hemorrhages at nearly all injection sites including controls. In addition, individual animals showed edema and/or round cell infiltration at the injection site. Although the incidence of the effects was similar at both the control and treatment injection sites, effects at the control site were probably due to the circulating concentrations of r-Hirudin. Clinical and histological findings were more prevalent in the initial study compared to the repeat studies (i.v. or perivenous). This difference, along with the restlessness observed following i.a. injection may have been related to a lower pH of the test article in the initial studies (pH = 3.8) versus the repeat studies (pH = 7.0).

The three local toxicity studies which utilized subcutaneous routes of administration showed the following: In the first study, single s.c. injection of r-hirudin produced unrest in 2 of the 4 animals (possibly due to low pH = 3.52). Doses $\geq$ 25 mg produced swelling, redness and hemorrhages at the injection sites and a dose-dependent increase in skin fold thickness (relative to mean control cutimetric values of 2.8 mm) was observed at all doses tested. Gross pathological findings included: bloody infiltrations in the s.c. tissue (most injection sites) and a hematoma in one animal at the 50 mg dose. Histological correlates of bloody edema and

subcutaneous hemorrhages were also observed at most injection sites. In the second study, like the first, r-Hirudin produced a dose-dependent increase in mean skin thickness at doses ≥ 12.5 mg/kg , with swelling and focal redness at the injection site in individual animals. Approximately half of the injection sites (including controls) showed gross findings of slight to moderate hemorrhagic infiltrations and histological correlates of hemorrhages in the subcutaneous tissue and diffuse round cell infiltration. Finally in the third study, subcutaneous injection of r-Hirudin at a dose 25 mg in a volume of 0.5 ml (dissolved in aqua ad injectabilia) produced a slight increase in mean skin thickness (16%, relative to mean control values of 3 mm), slight to moderate hemorrhages at the site of injection and a small nodule (an abscess) at the site of injection in one male. Although the development abscesses indicates incompatibility the relationship of the current finding to test article was considered equivocal since only a single instance of 10 animals tested was observed. Hemorrhagic effects were apparently due to pharmacodynamic properties of the drug and not as a result of local toxicity.

In conclusion, initial studies indicated that r-hirudin was not well tolerated following i.v.(25 mg in 0.5 ml) and perivascular (5 mg in 0.1 ml) injections and was incompatible after intra-arterial (25 mg in 0.5 ml) injection. In repeat studies in which r-hirudin was dissolved in aqua ad injectabilia, i.v. (25 mg; 0.5 ml), s.c. (12.5, 25, 37.5 and 50 mg in 0.5 ml) or (25 mg in 0.5 ml), intraarterially (25 mg in 0.5 ml) and perivascularly (5 mg in 0.1 ml) produced effects due to its pharmacodynamic activity following i.v. administration, but otherwise showed no evidence of a vascular irritation effect. The low pH of r-hirudin in the initial studies (i.e. pH = 3.8) may have contributed to the augmented irritant effects observed in the initial studies. A similar lack of local toxicity other than effects due to its pharmacodynamic activity were observed following s.c. injection.

Local Toxicity of r-Hirudin Following Intravenous, Intraarterial, Perivenous and Subcutaneous Administration in Rabbits (Report # 134.4-24, 134.4-24.1, 134.4-24.2 and 134.4-24.3)

Testing Laboratories: Behringwerke AG
Marburg, Germany

APPEARS THIS WAY
ON ORIGINAL

Study Started: January 30, 1995

Study Completed: February 1, 1995 and February 6, 1995 for study # 134.4-24.3.

GLP Requirements: A Statement of Compliance with OECD GLP regulation was included.

Animals: New Zealand white male rabbits.

APPEARS THIS WAY
ON ORIGINAL

Drug Batch No.: 114011

Methods: Rabbits (10/group) were given a single i.v. (25 mg/ 0.5 ml) i.a. (25 mg/0.5 ml) or p.v. (5 mg/0.1 ml) injection of r-hirudin in the ear. The other ear of rabbits received vehicle (0.9% saline) in similar fashion. Rabbits (10/group) were also given a single s.c. (25 mg/0.5 ml) injection of r-hirudin under the right lateral abdominal skin. Under the left lateral abdominal skin 0.9% saline was given in similar fashion. Five animals each were sacrificed on day 2 and 3 of the experiment and injection sites were examined microscopically.

Results: Post i.v. or i.a. dose, the injection sites had slight-moderate hemorrhages in the perivascular area (similar results were also seen after control injections). No abnormalities were seen when r-hirudin was given via p.v. or s.c. route. Hence, drug is "tolerable" after an i.v., i.a., p.v. or s.c. injection.

APPEARS THIS WAY
ON ORIGINAL

Local Toxicity of r-Hirudin After Repeat I.V. Injection in Rabbits (Study # 134.4-24.7)

Testing Laboratories: Behringwerke AG
Marburg, Germany

APPEARS THIS WAY
ON ORIGINAL

Study Started: April 3, 1995

APPEARS THIS WAY
ON ORIGINAL

Study Completed: April 14, 1995

GLP Requirements: A Statement of Compliance with OECD GLP regulations was included.

Animals: New Zealand white male rabbits. APPEARS THIS WAY ON ORIGINAL

Drug Batch No.: 114011

Methods: Rabbits (5/group) were given daily i.v. (into the ear vein) doses of r-hirudin for 7 consecutive days. Two control rabbits were given 0.9% saline into left ear under the same conditions. Rabbits were observed for up to 12 days and then sacrificed. All injection sites were examined microscopically.

Results: All rabbits which received r-hirudin developed a slight to severe reddening in the perivascular area during the first 7 days of the study period, and this finding was mostly reversible. Histopathological examinations of the injection sites revealed slight hemorrhages in the perivascular area in 2/5 rabbits without any inflammation. Hence, r-hirudin does not produce local irritation in rabbits.

APPEARS THIS WAY ON ORIGINAL

Antigenicity Studies:

Antigenicity Studies with r-Hirudin in Guinea Pigs (Report No. 134-03, 134-03.2, and 134.2-03)

Testing Laboratories: Behringwerke AG, Marburg

APPEARS THIS WAY ON ORIGINAL

Study Started: Report #134-03: 6/7/88; Report # 134-03.2: 10/12/88, and Report #134.2-03: 4/22/92.

APPEARS THIS WAY ON ORIGINAL

Study Completed: Report #134-03: 8/5/88; Report # 134-03.2: 12/7/88, and Report #134.2-03: 7/13/92.

GLP Requirements: A Statement of compliance with the GLP regulations and quality assurance unit was included in each study.

Drug Batch No.: U 001 and A 016

APPEARS THIS WAY ON ORIGINAL

Methods: The antigenicity of r-Hirudin was investigated in three studies conducted in guinea pigs. In the first study, groups of guinea pigs (N =5 or 10) were immunized (via s.c. injection) with either 0.2 or 1.0 mg r DNA-Hirudin dissolved in 0.5 ml isotonic saline + 0.5 ml complete Freund's Adjuvant (cFA) on days 0, 7, and 14. Two control groups likewise received either vehicle (0.5 ml isotonic saline) + 0.5 ml cFA or no treatment. In the second study (conducted without cFA), three groups of five Hartly female guinea pigs were immunized (via s.c. injection) with r-DNA-Hirudin at dose levels of 0.1, 1.0, and 5.0 mg/animal

dissolved in 0.5 ml isotonic saline on days 0, 7, and 14. A fourth group of control animals likewise received equal volume injections of isotonic saline alone. Finally in the third study (also conducted without cFA), two groups of 5 male guinea pigs were immunized subcutaneously with 1 or 5 mg of r-DNA-Hirudin dissolved in isotonic saline (1 ml) on days 0, 7, and 14. A third vehicle control group of (N = 2) were likewise administered isotonic saline (volume not indicated) alone. On Days 28 (studies 1 and 2) and on day 29 (study 3), all animals were challenged by i.v. injection of r-Hirudin (1.0 mg in 1 ml isotonic saline) followed by observation periods of 4 hours (Study 1) or 60 min (studies 2 and 3). Animals in the second study underwent a second challenge with 1 mg r-Hirudin (i.v.) at 14 days after the first again followed by a 60 min observation period. Finally, blood samples were also collected from guinea pigs in the third study on days 27 and 41 for determination of r-DNA-Hirudin specific and yeast (host cell antibodies) using ELISA testing.

Results: In the first study, all animals immunized with r-Hirudin showed severe anaphylactic reactions resulting in the death of 9 of 10 animals tested at the 0.1 mg level and in 5 of 5 animals tested at the 1.0 mg level. Although no effects were observed in the untreated control animals, vehicle control animals which received saline + cFA also showed restlessness and respiratory disorders (of unknown causes) occurring at after challenge and recovering 5-10 min later. In the second study (conducted without cFA in females), reactions were only seen in animals immunized with the 5 mg dose of r-Hirudin. At the first challenge reactions were limited to red ears in all animals tested. However at the second challenge, 2 animals died due to anaphylactic shock, 1 of 5 animals showed reversible shock-like symptoms, 1 of 5 animals showed only red ears and the fifth animal showed no effects. Finally, in the third study (conducted without cFA in males) only one of 5 animals immunized with the 5 mg dose showed respiratory disorders, lateral prostration and shock. In the third study, antibody titers were increased in 4 of 5 animals
and in all 5 animals
immunized with the 1.0 and 5 mg doses, respectively. In all cases, titers were further elevated following challenge at both immunization levels.

In conclusion, immunization with r-hirudin at doses of 0.1 and 1.0 mg/kg in combination with cFA resulted in severe anaphylactic reactions and death. However, unexplained pathological reactions (respiratory disorders) were also seen in the vehicle + cFA treated groups rendered the findings somewhat ambiguous. Thus, the Sponsor should repeat the study on the antigenic potential of rDNA-Hirudin in the presence of cFA in guinea pigs. In two other studies conducted in guinea pigs in the absence of cFA, challenge with r-Hirudin produced anaphylactic-like responses following immunization with high doses 5.0 mg/kg, but not with doses of 1.0 or less. However, increased antibodies toward r-hirudin were detected following immunization at both the 1.0 and 5 mg doses suggesting that r-hirudin induces humoral immunogenic responses in guinea pigs.

Antigenicity Study of r-Hirudin in Guinea Pigs (Study No. 134.14-09).

Testing Laboratory: Behringwerke Aktiengesellschaft
D 35001 Marburg, Germany

Compliance with Good Laboratory Practices and Quality Assurance Requirements: Statements of compliance were provided.

Study Started: January 11, 1996

Study Completed: May 15, 1996

Animals: Male and female approximately 4 weeks of age) Hartley guinea pigs.

Methods: Five groups of 10 guinea pigs each (5 males and 5 females) were subcutaneously immunized with 0.5 ml isotonic saline + 0.5 ml Freund's complete adjuvant (cFA)/animal (Group 1); 0.5 ml isotonic saline (Group 2); 0.1 mg r-hirudin dissolved in 0.5 ml of isotonic saline + 0.5 ml cFA/animal (Group 3); 1.0 mg r-hirudin dissolved in 0.5 ml isotonic saline + 0.5 ml cFA/animal (Group 4); and 1.0 mg r-hirudin dissolved in 0.5 ml isotonic saline (Group 5), respectively. Twenty-nine days later, all animals were challenged with intravenously administered r-hirudin (1 mg dissolved in 0.5 ml isotonic saline). Animals were observed for 24 h after challenge.

Results: There were no anaphylactic reactions to challenge in Groups 1 and 2. In Group 3, 4 males and 4 females died following challenge. In Group 4, 5 males and 4 females died following challenge. In Group 5, 1 male and 1 female exhibited excitation and sneezing following challenge; 1 male displayed respiratory distress and shock-like symptoms following challenge.

Thus, r-hirudin has antigenic potential when subcutaneously administered together with cFA. Less severe anaphylactic reactions occurred in animals immunized with r-hirudin alone.

Antigenicity of r-DNA-Hirudin Thrombin Complex in Guinea Pigs (immunization without Freud's Adjuvant) (Study # 124-03.1)

Testing Laboratories: Behringwerke AG
Marburg, Germany

Study Started: October 27, 1988

Study Completed: December 7, 1988 (report date)

GLP Requirements: A Statement of Compliance with GLP regulations was included.

Animals: 14-weeks old female Hartley guinea pigs (about 350 g).

Drug Batch No.: U001

Methods: Guinea pigs (5 females/group) were sensitized by s.c. administration of vehicle (0.9% saline), 0.03, 0.3 or 3.0 mg of rDNA thrombin-hirudin complex/animal once a week for 3 weeks (immunizations were performed without adjuvant). On day 28 animals were challenged by i.v. administration of 1 mg rDNA-hirudin. All animals were observed for 60 min following the challenge.

Results: Slight-moderate reaction (red ears and respiratory disorders) were seen in animals sensitized with rDNA-thrombin-hirudin complex, and no such reactions were seen in control group animals.

Antigenicity Studies with r-Hirudin in Rabbits (Report No. 134-04)

Testing Laboratories: Behringwerke AG, Marburg/Lahn

Study Started: July 12, 1993

Study Completed: May 13, 1994

GLP Requirements: A Statement of compliance with the GLP regulations and quality assurance unit was included in each study.

Animals: White New Zealand Rabbits; SPF; Ages: Males 14 weeks; Females 15 weeks

Drug Batch No.: U 001 and A 016

Methods: Two groups of rabbits (3/sex/group) repeatedly immunized with r-Hirudin [reconstituted with injectable water and suspended in a ratio of 1:2 complete Freund's Adjuvant (cFA; Group 1) or dissolved in a 0.05% solution of Aerosil; Group 2]. Group 1 rabbits were injected subcutaneous with r-Hirudin (5 mg + cFA) in a total volume of 2 ml/rabbit on study days 1 and 8 (cycle 1) and again on study days 22 and 29 (cycle 2). Group 2 rabbits were injected intravenously with r-Hirudin (5 mg + 0.05% Aerosil) in a total volume of 3 ml/rabbit for 5 consecutive days beginning on study day 1 (cycle 1) and again beginning on day 22 (cycle 2). Animals were evaluated daily for clinical signs of toxicity and body weights were determined on a weekly basis. Blood samples were collected on days: 8, 11, 15, 18, 22, 23, 24, 26, 29, 32, 36, 39, 43, 46, 50, 53, and 57 (Group 1) and on days 1, 2, 3, 5, 8, 11, 15, 18, 22, 23, 24, 26, 29, 32, 36, 39, 43, and 46 (Group 2) for determination of antibody formation. Antibodies against r-Hirudin were measured by indirect ELISA using hirudin coated plates and anti-rabbit-IgG antibody labeled with peroxidase and also by inverse using hirudin coated plates and biotinylated hirudin with peroxidase labeled streptavidin. No controls were included in the study.

Results: There was no clear indication of the induction of antibody formation following i.v. immunization as antibody levels were variable and remained below 80 ng/ml, with the exception of one animal #2003 which showed an increase in antibody levels and could possibly represent the onset of antibody formation. However, clear induction of antibody formation was observed following s.c. injection. Increases from base line values of 1-17 ng/ml began on day 11-15 and peaked around day 22, with peak values ranging . In all but on case, the maximal values measured by the were 2 to 8 fold lower than measurements obtained with the indirect assay. The exception, Animal # 1001 showed a clearly stronger signal with the on days 15 and 18, most likely representing an IgM antibody signal. However, no indication of IgM formation was evident in the other 5 animals tested. Injection of the second s.c. cycle of r-Hirudin resulted in bolstered peaks which occurred at day 29 and day 36 with maximal anti-r-Hirudin antibody levels which ranged from 400 µg/ml up to 1.65 mg/ml, with peak heights on both days being of similar magnitude. As was the case following the first cycle, antibody levels measured using the were on average 11 to 13 times less than those measured using the . Again however, one animal (#1005) showed a stronger signal using the on day 57 relative to measurements using the suggesting the induction of IgM antibodies.

In conclusion, r-Hirudin dissolved in and injected intravenously produced no clear induction of IgG or IgM antibody formation in rabbits. In contrast, definitive induction of antibody formation was observed in rabbits following subcutaneous injection of r-Hirudin suspended in cFA, with peaks occurring on days 22 and subsequent to the second cycle on days 29 and 36. Finally, 2 of 5 rabbits tested showed evidence of induction of IgM antibody formation. In addition to the aforementioned studies in Guinea pigs and rabbits, the Sponsor also indicated that tests for humural hirudin antibodies were conducted as an adjunct to the 3-month toxicity studies in monkey (Study No. 90.0762). However, the results from the latter study were not currently reported. Thus, the Sponsor should be asked to provide results from the humoral hirudin antibody evaluations conducted in the 3-month toxicity study in monkeys.

Antigenicity Study of r-Hirudin in Monkeys (Study No. 134.4-07.1).

Testing Laboratory: Behringwerke Aktiengesellschaft
D 35001 Marburg, Germany

Compliance with Good Laboratory Practices and Quality Assurance Requirements: Statements of compliance were provided.

Study Started: May 29, 1995

Study Completed: June 5, 1996

Animals: Male cynomolgus monkeys.

Methods: Eight monkeys were subcutaneously immunized with emulsifed r-huridin (2.0 mg/kg) in Freund's complete adjuvant (cFA; 0.5 ml) into the right abdomino-inguinal region on Day 1 and the left abdomino-inguinal region on Day 8. All animals were challenged with intravenously administered r-hirudin (0.4 mg/kg) + an intravenous infusion of r-hirudin (0.15 mg/kg/h) for 6 h on Days 22 and 43. Before start of the infusion, the animals were narcotized with and (ratio of 1:1; 0.5 ml/animal).

Animals were observed daily for clinical signs of toxicity up to Day 95. Body weights were recorded on Day 1 and on a weekly basis thereafter.

Activated partial thromboplastin times (APTTs) were determined before start of treatment; and 30 min after bolus injection of r-hirudin, 5 h after start of r-hirudin infusion and 30 min after end of r-hirudin infusion on Days 22 and 43.

Blood samples for determination of plasma r-hirudin concentrations were collected before start of treatment; and 30 min after bolus injection of r-hirudin, 5 h after start of r-hirudin infusion and 0.5, 3 and 6 h after end of r-hirudin infusion on Days 22 and 43.

Blood samples for determination of IgG, IgM and IgE antibody titres were collected on Days 1, 18, 20 and 22, twice weekly for the subsequent 3 weeks, on Day 43, and twice weekly thereafter until Day 95.

All animals that died during the study and all surviving animals were subjected to gross pathological and histopathological examinations.

Results:

1. Observed Effects: Swelling of inguinal lymph nodes was observed in all animals from Day 15 to Day 43 of treatment. From Day 22 and thereafter, local irritations at sites of injection were seen. There were no other clinical signs of toxicity.

2. Mortality: Six animals died from 30 min to overnight after start of r-hirudin infusion on Day 43. Four of these animals exhibited retching and vomiting during infusion. The 2 remaining animals survived until the end of the study (Day 95).

3. Body Weights: There were transient and modest body weight losses after intravenous infusions of r-hirudin.

4. APTTs: Mean APTT was 26.9 min on Day 1 before initiation of treatment.

Mean APTT was 51.9 min before start of treatment on Day 22. Mean APTTs were 109.3, 65.3 and 68.1 at 30 min after bolus injection of r-hirudin, 5 h after start of r-hirudin infusion, and 30 min after end of r-hirudin infusion, respectively, on Day 22.

Mean APTT was 44.3 min before start of treatment on Day 43. Mean APTTs were 51.6, 73.5 and 69.6 at 30 min after bolus injection of r-hirudin, 5 h after start of r-hirudin infusion, and 30 min after end of r-hirudin infusion, respectively, on Day 43.

5. Plasma concentrations of r-hirudin and antibody titres: As shown in the following table, plasma levels of r-hirudin at 5 h after the start of infusion ranged on Day 22 and from on Day 43

. Peak antibody titres up to 50 and 360 µg/ml for IgG and IgM, respectively, were detected. Antibody titres for IgE were relatively low (10 µg/ml or less).

Plasma concentrations of r-hirudin and antibody titres

Animal No.	Plasma level of r-hirudin (ng/ml)		Peak IgG (μg/ml)	Peak IgM (μg/ml)	Peak IgE (μg/ml)
	Day 22	Day 43			
1	1991	3052	30	19	<6
2	2278	4140	50	162	9
3	2198	-	29	116	10
4	9869	5083	25	360	9
5	2315	8361	14	257	<6
6	12200	9096	34	202	9
7	740	1748	28	331	10
8	1044	9098	47	101	9

6. Gross Pathology: There were local irritations at subcutaneous injection sites in all animals. Patchy lungs were observed in 3/8 animals; patchy kidneys in 1/8 animals.

7. Histopathology: As shown in the following table, there were histopathological lesions in injection site, lymph nodes, lungs, kidneys, heart and liver.

Incidence of histopathological lesions

Organ/lesion	Incidence of lesion
Injection site Granulomatous inflammation with necrotic areas and diffuse activity of giant cells	5/7
Lymph nodes Granulomatous inflammation with necrotic areas and diffuse activity of giant cells Sinus histiocytosis Hypervascularization	2/7 2/7 1/7
Lungs Granulomatous inflammation Giant cell activity Fibrosis Edema Hemorrhage Emphysema Oedematization	6/7 5/7 2/7 1/7 2/7 2/7 2/7
Kidneys Acute interstitial nephritis Granulomas Giant cell activity PAS-positive and eosinophile casts in tubuli	4/7 2/7 3/7 2/7
Heart Multifocal inflammation in septum	1/7
Liver Necrosis Round cellular infiltration	2/7 3/7

In summary, r-hirudin challenge in monkeys immunized with r-hirudin in cFA produced death and histopathological lesions in injection site, lymph nodes, lungs, kidneys, heart and liver. Furthermore, plasma levels of r-hirudin appeared to be elevated in the presence of anti-hirudin antibodies.

Follow-up to Antigenicity Study of r-Hirudin in Monkeys (Study No. 134.4-07.3).

Testing Laboratory: Behringwerke Aktiengesellschaft
D 35001 Marburg, Germany

Compliance with Good Laboratory Practices and Quality Assurance Requirements: Statements of compliance were provided.

Study Started: August 7, 1995

Study Completed: January 19, 1996

Animals: Male cynomolgus monkeys.

Methods: In order to evaluate the assumption that cFA played a large role in the results of the antigenicity study in monkeys, the present study was performed exactly as the previous antigenicity study, with the exception that subcutaneously administered r-hirudin was given without cFA. Thus, eight monkeys were subcutaneously administered r-huridin (2.0 mg/kg) into the right abdomino-inguinal region on Day 1 and the left abdomino-inguinal region on Day 8. All animals were challenged with intravenously administered r-hirudin (0.4 mg/kg) + an intravenous infusion of r-hirudin (0.15 mg/kg/h) for 6 h on Days 22 and 43. Before start of the infusion, the animals were narcotized with
(ratio of 1:1; 0.5 ml/animal).

Animals were observed daily for clinical signs of toxicity up to Day 58. Body weights were recorded on Day 1 and on a weekly basis thereafter.

Activated partial thromboplastin times (APTTs) were determined before start of treatment; and 30 min after bolus injection of r-hirudin, 5 h after start of r-hirudin infusion and 30 min after end of r-hirudin infusion on Days 22 and 43.

Blood samples for determination of plasma r-hirudin concentrations were collected before start of treatment; and 30 min after bolus injection of r-hirudin, 5 h after start of r-hirudin infusion and 0.5, 3 and 6 h after end of r-hirudin infusion on Days 22 and 43.

Blood samples for determination of IgG, IgM and IgE antibody titres were collected on Days 1, 18, 20 and 22, twice weekly for the subsequent 3 weeks, on Day 43, and twice weekly thereafter until Day 95.

Results:

1. Observed Effects: Enlargement of both inguinal lymph nodes was seen in 4 animals from Day 25 to Day 31. One animal exhibited hematoma and nodule formation at site of injection from Day 8 to Day 14. One animal had diarrhea on Day 24. There were no other clinical signs of toxicity.

2. Mortality: There were no deaths.

3. Body Weights: There were transient and modest body weight losses after intravenous injection of r-hirudin.

4. APTTs: Mean APTT was 25.5 min on Day 1 before initiation of treatment.

Mean APTT was 29.2 min before start of treatment on Day 22. Mean APTTs were 54.0, 58.6 and 48.6 at 30 min after bolus injection of r-hirudin, 5 h after start of r-hirudin infusion, and 30 min after end of r-hirudin infusion, respectively, on Day 22.

Mean APTT was 28.3 min before start of treatment on Day 43. Mean APTTs were 57.4, 64.0 and 54.2 at 30 min after bolus injection of r-hirudin, 5 h after start of r-hirudin infusion, and 30 min after end of r-hirudin infusion, respectively, on Day 43.

5. Plasma concentrations of r-hirudin and antibody titres: As shown in the following table, plasma levels of r-hirudin at 5 h after the start of infusion ranged between 294 and 1400 ng/ml on Day 22 and from 698 to 3747 ng/ml on Day 43 (as measured by ELISA). Antibody titres for IgG and IgM were below threshold levels of detection.

Plasma concentrations of r-hirudin and antibody titres

Animal No.	Plasma levels of r-hirudin (ng/ml)		IgG (µg/ml)	IgM (µg/ml)
	Day 22	Day 43		
1	860	744	<0.94	<14.4
2	1400	702	<0.94	<14.4
3	1205	3747	<0.94	<14.4
4	581	1114	<0.94	<14.4
5	294	698	<0.94	<14.4
6	1102	1556	<0.94	<14.4
7	916	1032	<0.94	<14.4
8	1194	860	<0.94	<14.4

In summary, the absence of detectable antibody titres for IgG and IgM were associated with relatively low plasma levels of r-hirudin. In contrast, it was previously shown that plasma levels of r-hirudin were elevated in the presence of anti-hirudin antibodies. Thus, induction of anti-hirudin antibodies influences the pharmacokinetics for r-hirudin.

Dermal Sensitization Test
(Study # 95.0151)

Study Started: May 2, 1995

Study Completed: June 2, 1995

GLP Requirements: A Statement of Compliance with GLP regulations was included.

Animals: white female guinea pig (285 g).

Drug Batch No.: 118011

Methods: Ten female guinea pigs were given two intracutaneous injection of 0.1 ml of 50% Freund's Complete Adjuvant, r-hirudin (0.1 ml of 50 mg/ml = 5 mg) and r-hirudin with Freund's adjuvant (1:1) at 3 sites on dorsal area. The animals of control group (n=5) were given similar treatment except drug was replaced with saline. One week later, animals were dermally exposed for 48 hr (under occlusive bandage) with 0.5 ml of 50 mg/ml r-hirudin or saline. The skin reaction were observed. Two weeks later the animals were dermally challenged over a period of 24 hr by applying 0.5 ml of 20% (5 mg) solution of r-hirudin. The skin reaction was observed at 24 hr after the challenge.

Results: No delayed hypersensitivity reaction was seen in r-hirudin treated guinea pigs. However, during the intradermal induction treatment the injections containing Freund's complete adjuvant evoked severe erythema and edema in the treated as well as in the control group.

Allergic Reactions to Yeast-Derived Contaminating Proteins Originating From Working Seed Cells For r-Hirudin (Study No. P-248).

Animals: Male and female guinea pigs.

Testing Laboratory: Behringwerke Aktiengesellschaft
D 35001 Marburg, Germany

Compliance with Good Laboratory Practices and Quality Assurance Requirements: Statements of compliance were provided.

Study Started: September 5, 1995

Study Completed: January 2, 1996

Methods: Three groups of 10 guinea pigs each (5 males and 5 females) were subjected to subcutaneous immunization with Yeast Derived Contamining Protein Mock (YCP)-Preparation (1 ml/animal), YCP (1 ml/animal), and r-hirudin (1.0 mg/animal), respectively, on Days 1, 8, 15 and 22; and challenged with intravenously administered YCP (1 ml/animal), r-hirudin (1.0 mg/animal) and r-huridin (1.0 mg/kg), respectively, on Day 37. Animals were observed for 24 h after challenge.

Results: As shown in the following table, when guinea pigs were immunized with s.c. YCP and challenged with either i.v. r-hirudin or i.v. YCP, there were allergic reactions in 50% of challenged animals. Severity of the allergic reaction ranged from moderate to severe. When animals were immunized with s.c. r-hirudin and challenged with i.v. r-huridin, there were moderate allergic reactions in 20% of the challenged animals.

Thus, yeast-derived contaminating proteins originating from working seed cells are potentially allergenic and may have contributed to allergic reactions associated with r-hirudin administration in earlier studies.

Immunization	Challenge	*Score (# of animals)			
		0	1	2	3
YCP (1 ml/animal, s.c.)	r-Hirudin (1.0 mg/animal, i.v.)	5	4	1	0
YCP (1 ml/animal, s.c.)	YCP (1 ml/animal, s.c.)	5	3	1	1
r-Hirudin (1.0 mg/animal, i.v.)	r-Hirudin (1.0 mg/animal, i.v.)	8	2	0	0

*0=No reaction, 1=excitation, sneezing, 2=respiratory distress,

Antigenicity of the Yeast Protein T3329 in Sheep
(Study # 9725)

Study Started: Not given.

Study Completed: January 18, 1990 (report date)

Methods: Ten sheep were given s.c. injection of 1 mg yeast fragment T3329 (mix with saline and complete Freund's adjuvant) at several sites in "close proximity of the regional lymph nodes". Booster administrations of 0.5 mg T3329 in combination with incomplete Freund's adjuvant were given at 10-day intervals. Ten days after the third booster, blood samples were taken from the jugular vein for measuring anti T3329 antibody titres (results were not submitted in the report). Five animals having the highest antibody titres were selected for intracutaneous test. T3329 (1 mg, 10 μg and 100 ng) was administered intradermally on one side of the abdomen and r-hirudin (5 mg, 500 μg, and 50 μg) was administered on opposite side. Intradermally 0.5 ml of normal saline was injected into one section of abdomen as negative control. Skin were evaluated at 1, 2, 3, 24 and 48 hr after the challenge.

Results: No skin reaction was seen when r-hirudin was administered, however, intradermal administration of T3329 (1 mg) produced edema and swelling at the injection sites. Similar results were seen in repeat experiment when 0.5 mg of T3329 was used. Data indicated that r-hirudin (lot # not given) does not contain any yeast impurities and/or the amount of contaminating carrier proteins is below threshold dose i.e. 500 μg.

APPEARS THIS WAY
ON ORIGINAL

Proposed Text of the Labeling for r-Hirudin:

The label is according to 21 CFR, 201.50 Subpart B (April 1, 1996). However, the following changes should be incorporated.

1. **Animal Data**

Sponsor's Version:

Reproduction Toxicity

A developmental study showed an embryotoxic action of Refludan® (i.e. an increased post implantation loss) at 30 mg / kg / day in rabbits (i.e. 7.5 times the human standard dose).

In rats, the administration of Refludan® 30 mg / kg / day during the peri- and postnatal period, resulted in increased maternal mortality at parturition. At this dose the survival of the pups was slightly reduced and the incidence of pups with dilatation of the renal pelvis was elevated.

Mutagenicity / Carcinogenicity

Refludan® was not mutagenic in bacterial and somatic gene mutation assays (Ames test, HGPRT test with V 79 cells of the Chinese hamster in vitro), in the chromosomal aberration assay in Chinese hamster V 79 cells and in a micronucleus assay in the mouse. Carcinogenicity studies were not performed.

Evaluation:

The text is not according to 21 CFR, 201.50, Subpart B (April 1, 1996).

Proposed Version:

Carcinogenesis, Mutagenesis, Impairment of Fertility:

No long-term studies in animals have been performed to evaluate carcinogenic potential of Lepirudin. Lepirudin was not genotoxic in the in vitro chromosomal aberration test in A549 mammalian cell line, in V79 Chinese hamster cells, HGPRT forward gene mutation assay in V79 cells and mouse micronucleus test. Lepirudin at i.v. doses up to 30 mg/kg/day (180 mg/m^2/day, 1.2 times the recommended maximum human dose [0.4 mg/kg i.v. bolus + 0.15 mg/kg/hr = 4 mg/kg/day = 148 mg/m^2/day] based on body surface area) was found to have no effect on fertility and reproductive performance in rats.

2. Pregnancy

Sponsor's Version:

Pregnancy category C. **Refludan® has been shown to have an embryotoxic effect in rabbits and rats when given in doses 7.5 times the human dose (see Animal Data below). There are no adequate and well-controlled studies in pregnant women. Refludan® should be used during pregnancy only if the potential benefit justifies the potential risk to the fetus.**

Evaluation:

In i.v. Segment II. teratology study in rats, doses of 1, 10 and 30 mg/kg/day were used. Neither embryotoxic nor teratogenic effects at doses up to 30 mg/kg/day were observed.

In i.v. Segment II. teratology study in rabbits, doses of 1, 10 and 30 mg/kg/day were used. No teratogenic effects at doses up to 30 mg/kg/day was observed. However, sponsor indicated that highest tested dose was embryotoxic (increased early resorptions with reduced live litter sizes) in rabbits. Hence sponsor classified as pregnancy category C. We disagree with the sponsor classification. In rabbits, increase in early resorption at high dose was seen in only 3 out of 15 rabbits (# H30050, # H30059 and # H 30063). If we exclude these animals from analysis then no embryotoxicity was evident. Therefore, in light of the above results, the classification of the pregnancy should be category B.

Proposed Version:

Pregnancy: Teratogenic effects. Pregnancy Category B.

Reproduction studies have been performed in rats at i.v. doses up to 30 mg/kg/day (177 mg/m^2/day, 1.2 times the recommended maximum human dose based on body surface area) and in rabbits at i.v. doses up to 30 mg/kg/day (257.1 mg/m^2/day, 1.7 times the recommended maximum human dose based on body surface area) and have revealed no evidence of impaired fertility or harm to the fetus due to Lepirudin. There are, however, no adequate and well controlled studies in pregnant women. Because animal reproduction studies are not always predictive of human response, this drug should be used during pregnancy only if clearly needed.

SUMMARY AND EVALUATION:

Lepirudin (r-hirudin) is a recombinant hirudin derived from yeast cells which were transfected with an expression vector containing hirudin gene. It is identical to natural hirudin except the two N-terminal amino acids, leucine at position 1 and threonine at position 2, are substituted for the two N-terminal amino acids, valine-1 and valine-2 or isoleucine-1 and threonine-2 of natural hirudin isoforms. Additionally, r-hirudin is not sulfated on the tyrosine at position 63. r-Hirudin binds selectively to thrombin and does not require the presence of antithrombin III cofactor. It dose dependently prolonged activated partial thromboplastin time (APTT) and thrombin time (TT) in various species (rats, dogs, cats and monkeys). It has antithrombotic activity in various models of thrombosis.

In support of the new drug application for Lepirudin (r-hirudin), sponsor had submitted preclinical data from the pharmacology studies; absorption, distribution, metabolism and excretion (ADME) studies in rats, rabbits, dogs and monkeys; acute toxicity studies in mice, rats and monkeys; 3-day (i.v. bolus + i.v. infusion), 1-month (i.v. and s.c.) and 13-week (i.v.) and 3-month (s.c.) toxicity studies in rats; 3-day (i.v. bolus + i.v. infusion), 15-day (i.v. bolus + i.v. infusion), 1-month (i.v.), 13-week (i.v.) and 3-month (s.c.) studies in monkeys; i.v. Segment I fertility and general reproductive performance studies in rats, i.v. Segment II teratology studies in rats and rabbits and i.v. Segment II/III perinatal and postnatal studies in rats; genotoxicity studies: Ames test, in-vitro UDS assay in A549 mammalian cell line, in-vitro chromosomal aberration test in V79 Chinese hamster cells, HGPRT forward gene mutation assay in V79 cells and mouse micronucleus test and special toxicity studies: local tolerance studies in rabbit after i.v., i.a., p.v. and s.c. dose, antigenicity studies in guinea pigs, rabbits and monkeys, dermal sensitization test, allergic reaction to yeast-derived contaminating proteins from r-hirudin and antigenicity of the yeast protein T3329 in sheep.

Absorption, distribution, metabolism and excretion studies were conducted in rats, rabbits, dogs and monkeys. Irrespective of the species $t_{1/2}$ ß is <2 hr (man = 1.3 hr), clearance ranged from

used) and volume of distribution ranged
In rats, administered radioactivity was distributed throughout the body, and levels in kidneys, urinary bladder and thyroid were higher than that seen in plasma. The levels of radioactivity increased with time in thyroid whereas they decreased in the remaining organs. The increase level of radioactivity in thyroid is mainly due to deiodination of the labeled drug and to storage of iodide in thyroid. r-Hirudin and/or its metabolites crosses

placental barrier. In rats seven urinary metabolites were identified (I-50, I-52, I-56, I-53, I-51, 2-51 and I-61). In rats, I-50 and I-52 were major metabolites. In vitro, r-hirudin biotransformed rapidly in kidney 9000 g fraction of rat, monkey and man. The biotransformation in kidney fraction from monkey and man were similar and only I-62 (monkey), I-63 and I-64 were identified (presumable breakdown product of carboxypeptidase). Irrespective of the species, excretion is mainly via renal route and fecal excretion is negligible.

Acute toxicity studies were conducted in mice, rats and monkeys. The highest tested dose did not produce any mortality or signs of toxicity in mice (1000 mg/kg [i.v.] or 1250 mg/kg [s.c.]), rats (1000 mg/kg, i.v.) and monkeys (100 mg/kg, i.v.). However, s.c. acute toxicity study in rats, 50 mg/kg was the highest non-lethal dose (100 mg/kg was the lethal dose). Hematomas and hemorrhages were seen at the injection site in rats after s.c. dose.

In i.v. bolus plus i.v. infusion toxicity study in rats, animals were given a single i.v. bolus dose of 0.2 or 0.4 mg/kg of hirudin followed by a 72-hour infusion of 0.10 or 0.15 mg/kg/hr of hirudin respectively. In this study highest tested dose (0.4 mg/kg, i.v. bolus + 0.15 mg/kg/hr for 72 hr i.v. infusion) was the no effect dose.

In 1-month i.v. toxicity study in rats, doses of 10 and 100 mg/kg/day were used. Local bleeding and necrosis (1 out of 10 rats), increased spleen weights , decreased erythrocytes (31%), hemoglobin (27%) and hematocrit (26%) values were seen in high dose treated females. Histopathological examinations revealed sinus catarrh in the regional lymph nodes (males: control = 0/5, low dose = 1/5, and high dose = 5/5; females: control = 0/5, low dose = 4/5 and high dose = 5/5). One high dose treated female had evidence of increased erythropoiesis in the bone marrow and extramedullary hematopoiesis in the liver.

In 13-week i.v. toxicity study in rats, doses of 1, 10 and 100 mg/kg/day were used. In this study no target organ of toxicity was identified. Mortalities were seen in mid and high dose treated rats. The lowest tested dose was the well tolerated dose.

In 1-month s.c. toxicity study in rats, doses of 1, 10 and 100 mg/kg/day were used. In this study no target organ of toxicity was identified. Mortalities were seen in mid and high dose treated rats. The lowest tested dose was the well tolerated dose.

In 3-month s.c. toxicity study in rats, doses of 0.4, 2.0 and 10.0 mg/kg/day were used. The highest tested dose produced deaths. Mid dose produced slight increase in thrombin times (males = 9.5% and females = 14.4%), reticulocytes counts (males = 106% and females = 15%) and mild hematoma at the injection sites. Lowest test dose can be considered as well tolerated dose since it produced only increase in reticulocyte counts in males (135%) and hemorrhages at the injection sites.

In i.v. bolus plus i.v. infusion toxicity study in monkeys, animals were given a single i.v. bolus dose of 0.2 or 0.4 mg/kg of hirudin followed by a 72 hr infusion of 0.10 or 0.15 mg/kg/hr of hirudin respectively. Dose related increase in APTT was seen in treated monkeys. This finding is related to the pharmacological effect of the drug. Hence, the highest tested dose was the no effect dose.

In 15-day i.v. (bolus + infusion) toxicity study in monkeys, animals were given a single i.v. bolus dose of 0.4 mg/kg of r-hirudin followed by a 14-day continuous infusion of 0.15 mg/kg/hr. No treatment related toxicities were seen. In line with pharmacological activity of the drug, prolongation of APTT was seen in treated males (APTT: control = 21.7 sec and treated = 23.8 sec).

In 1-month i.v. toxicity study in monkeys, doses of 0.1, 1 and 10 mg/kg/day were used. Dose dependent increase in clotting times were seen in treated monkeys (males: control = 254 sec, low dose = 349 sec, mid dose = 1566 sec and high dose = 3.5 hr; females: control = 171 sec, low dose = 630 sec, mid dose = 1976 sec and high dose = >3.5 hr), when measured at 10 min after drug administration on day 30 of the study. This finding is considered to be due to the pharmacodynamic action of r-hirudin.

In 13-week i.v. toxicity study in monkeys, doses of 1, 10 and 30 mg/kg/day were used. Mortalities were seen in mid and high dose treated monkeys. The lowest tested dose was the well tolerated dose since it only produced slight increase in APTT (males: control = 21.4 sec, low dose = 23.5 sec, and females: control = 21.8 sec and low dose = 22.1 sec).

In 3-month s.c. toxicity study in monkeys, doses of 0.3, 3 and 30 mg/kg/day (given in two divided doses) were used. The highest tested dose was lethal. No target organ of toxicities were identified. Low dose level can be considered as well tolerated dose since it only produced some s.c. bleeding at the injection sites.

Mortalities seen in the above mentioned toxicity studies in rats and monkeys were due to exaggerated pharmacodynamic effects of the drug (bleeding, anemia, hemorrhages and hematomas).

In i.v. Segment I. fertility and general reproductive performance study in rats doses of 1, 10 and 30 mg/kg/day were used. The male rats were treated from 64 days prior to mating, throughout the mating phase and until they were sacrificed. Females were treated from 15 days prior to mating, throughout mating and up to day 7 of gestations. There were no abnormal effects on the fertility and mating performance of the treated male and female rats at i.v. doses up to and including 30 mg/kg/day of r-hirudin.

In i.v. Segment II. teratology study in rats, doses of 1, 10 and 30 mg/kg/day were used. No teratogenic effects at doses up to 30 mg/kg/day were observed.

In i.v. Segment II. teratology study in rabbits, doses of 1, 10 and 30 mg/kg/day were used. No teratogenic effects at doses up to 30 mg/kg/day was observed.

In i.v. Segment II/III. perinatal and postnatal study in rats, doses of 1, 10 and 30 mg/kg/day were used. The highest tested dose produced maternal toxicity (11/25 dams died/killed in delivery and early post-partum period). However, no significant adverse effect on reproductive parameters were seen in rat following i.v. administration of up to 30 mg/kg/day of r-hirudin during perinatal and postnatal period. No treatment related macroscopic abnormalities were seen in females which died, nor in scheduled sacrificed animals.

No mutagenic potential was demonstrated when r-hirudin was tested in 5 different tests: Ames test, in vitro UDS test in A549 mammalian cell line, in vitro chromosomal aberration test in V79 Chinese hamster cells, HGPRT forward gene mutation assay in V79 cells and mouse micronucleus test.

Local tolerance studies were performed in rabbits after i.v., i.a., p.v. and s.c. dose. Perivascular redness and/or focal bleeding were seen in rabbits treated intravenously. No abnormalities were seen at the injection sites when r-hirudin was given via s.c. route. Slight hemorrhages and/or perivascular reddening were seen when drug was administered via i.a. or p.v. route. The local effects seen in rabbit are related to pharmacologic effect of the drug, otherwise showed no evidence of vascular irritation.

Less severe anaphylactic reactions upon challenge with i.v. dose of r-hirudin were seen in guinea pig when immunized with r-hirudin alone. However, severe anaphylactic reactions including deaths were seen in guinea pigs when immunized with r-hirudin in combination with complete Freund's adjunctive (cFA). r-Hirudin in the presence of cFA also produced severe dermal reactions in guinea pigs (r-hirudin in the absence of cFA did not produce allergic reaction). Studies in sheep failed to reveal the presence of liprudin-associated yeast impurities. Data indicated that r-Hirudin has antigenic potential.

Sponsor has adequately characterized r-hirudin and conducted sufficient preclinical toxicity studies in different species. Lepirudin(r-hirudin) is indicated for anticoagulation in adult patients with heparin-associated thrombocytopenia (HAT) type II and thromboembolic disease. From a preclinical standpoint the application is approvable.

The label is according to 21 CFR, 201.50 Subpart B (April 1, 1996), however, it needs minor changes in the text as outlined in the review portion.

APPEARS THIS WAY
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RECOMMENDATION:

From a preclinical standpoint the application is approvable. Sponsor should be asked to change the labeling as outlined in the review portion.

/S/ 5/8/97

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